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TITLE: The Role of Human Spectrin SH3 Domain Binding Protein 1 (HSSH3BP1) in Prostatic Adenocarcinoma

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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

Prostate cancer is one of the leading causes of cancer-related deaths in the United States and a leading diagnosed cancer in American men. Newly diagnosed cases of prostate cancer approach rapidly the number of 200, 000 cases per year. Genetic alterations of tumor suppressor genes are one of the most common causes of prostate cancer tumorigenesis. Our group identified Hssh3bp1 as a candidate prostate tumor suppressor gene. In this research we are testing the tumor suppressor function of the candidate gene in prostatic adenocarcinoma using *in vitro* and *in vivo* assays. Expression of Hsshb3p1 significantly decreased growth of LnCAP cells *in vitro* suggesting that Hssh3bp1 may be critical for regulation of growth of prostate tissue. In separate experiments we established Hsshb3p1 as a substrate of Abl tyrosine kinase, a known oncogene. Further work is proposed work to develop a detail understanding what is the mechanism of growth regulation by Hssh3bp1 and Abl kinase. It is hoped that this work will help us to understand prostate cancer tumorigenesis and ultimately lead to better diagnosis and therapy strategies.

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Introduction

Prostate cancer is one of the leading causes of cancer-related deaths in the United States (over 41, 000 per year) and a leading diagnosed cancer in American men (43% of all diagnosed cancer in men). Newly diagnosed cases of prostate cancer approach rapidly the number of 200, 000 cases per year. Genetic alterations of tumor suppressor genes are one of the most common causes of neoplastic transformation leading to tumorigenesis including prostate cancer tumorigenesis. Inactivation of one or more tumor suppressor genes is thought to be the most common cause of prostatic adenocarcinoma. Our group identified such candidate tumor suppressor gene. The gene was originally named Hssh3bp1 for its binding properties to spectrin SH3 domain (*human spectrin SH3 domain binding protein 1*).

In this research we propose to test the tumor suppressor function of a candidate gene in prostatic adenocarcinoma using *in vitro* and *in vivo* assays. The work is directed at understanding what is the mechanism of loss of hssh3bp1 expression in prostatic cells lines and tumors, and will test potential tumor suppressive role of hssh3bp1 in nude mice. Hssh3bp1 is a potential regulator of macropinocytosis. Macropinocytosis can be upregulated by growth factors, which in turn promote tumor growth; we propose that Hssh3bp1 is a negative regulator of macropinocytosis and cell growth. To learn more about possible mechanisms of Hssh3bp1 tumor suppressor function we will determine whether Hssh3bp1 mutations affect macropinocytosis of prostate cells and determine molecular events underlying this effect. Although it is possible that Hssh3bp1 is not involved in biogenesis of prostate cancer, after completion of the proposed work we will know more about the function of the protein in human prostate. On the other hand, with the identification of hssh3bp1 as a tumor suppressor gene in prostate cancer, it is likely to lead to subsequent hypotheses and research on the hssh3bp1 role in prostate tumorigenesis. This, in turn, is likely to lead to a better diagnosis, treatment, and possibly prevention of this deadly disease.

Body

The following are the aims of proposal as defined in the original Statement of Work:

Aim 1. To determine whether Hsshb3p1 is not expressed in some prostate tumors due to presence of mutations.

a. Search for mutations of the hssh3bp1 cDNA and gene in prostate tumor cell lines and primary prostate tumors (30 cases).
b. Determine pattern of hssh3bp1 expression in primary prostate tumors. Correlate the pattern of hssh3bp1 expression with the tumor grade and stage (100 cases).

Aim 2. To determine whether the Hssh3bp1 gene carries a tumor suppressor function *in vivo.*

a. Evaluate tumorigenicity of prostate cell lines containing mutated hsshb3p1 in athymic nude mice and in soft agar assay. Evaluate the tumorigenecity of cell lines transfected with the hssh3bp1 antisense plasmids in athymic nude mice and in soft agar assay.
b. Identify a region in hssh3bp1 responsible for the tumor suppression function.

Aim 3. To identify a potential mechanism and a signal transduction pathway involved in the tumor suppression function of Hssh3bp1.

a. Determine the role of hssh3bp1 mutations in macropinocytosis of prostate cell lines.
b. Determine the role of growth factors, PI3-kinase, and the 200-kDa spectrin-like protein in the function of hssh3bp1.

We have initiated the work towards all three Aims of the grant application.

Progress towards Aim 1.

The rationale for these experiments is that if Hsshb3p1 carries tumor suppressor function the gene mutations inactivating its function must exist in primary tumors and in tumor cell lines. Although loss of the hssh3bp1 expression may be due to other possibilities including downregulation of a signal transduction pathway(s) involving the gene in the prostate we will specifically search for mutations of Hsshb3p1 because this suggests a tumor suppression function.

We have initiated the collection of the prostate tissue material from the local hospital (St. Vincent's Hospital, Staten Island, NY). We have collected 11 specimen up to date. We in the process of preparing the Hssh3bp1 cDNA from these specimen to be characterized by sequencing (Aim 1a).

Progress towards Aim 1b: we re-evaluated the expression studies of Hssh3bp1 in prostate tissue by immunochemistry. We feel that availability of prostate tissue arrays may provide us with much better standardized tissue material (i.e. all tumor cases with non-tumor controls are on the same slide) than studies of tissue from various sources that we proposed. Therefore we are in the process of purchasing a tissue array with

100 tumor cases with case-matched non-tumor controls. The array slides will be funded from our institutional support. The goals of this Aim remain unchanged.

Progress towards Aim 2.

The rationale for these experiments is to test the hypothetical tumor suppressor function of Hssh3bp1 by complementation assays (Aim 2a). We hypothesized that tumorigenicity of some prostate cell lines is due to inactivation of Hssh3bp1 function. We determined that LnCaP cell lines, ATCC CRL-10995 and -1740 contain an exon-skipping Hssh3bp1 mutation (Macoska *et al.*, 2001). Thus it is possible that Hssh3bp1 function is impared in these cell lines. The goal of the complementation experiments is to transfect a correct copy of Hssh3bp1 gene to cells, restore the gene expression, and examine whether this will suppress malignant phenotype of tumorigenic cells. The experimental plan included establishment of stable clones, testing their growth characteristic by growth assay and colony formation in soft agar (*in vitro* assays) as well as testing of their malignant phenotype by tumorigenicity studies in nude mice (*in vivo* assay).

Establishement of prostate cancer cell lines stably transfected with Hssh3bp1.

We used LnCaP (CRL-1740) for the Hssh3bp1 transfection experiments. Isoform 2 of Hssh3bp1 was used for transfection because it is one of the major forms of Hssh3bp1 expressed in primary prostate cells (data not shown). Initially, ten clones expressing recombinant Hsshb3p1 cDNA and resistant to neomycin (the selection antibiotic) were selected. From these ten clones, however, only two clones, NG18-1 and NG18-10, survived following the selection protocol. Expression of the recombinant cDNA was monitored as described in Fig. 2. At the same time, a mock control cell line, N3G-1, expressing the recombinant vector without the Hssh3bp1 cDNA was selected under the same selection protocol. In the growth assay clones expressing Hssh3bp1 showed significant reduction of growth (about one third) in comparison to the mock control (Fig. 1). In addition, both clones expressing the recombinant Hssh3bp1 cDNA had very similar growth characteristics. These data strongly suggest that Hssh3bp1 plays a growth-inhibiting role in LnCaP cells. The developed cell lines will serve as model system to determine potential mechnism of Hssh3bp1 growth control function.

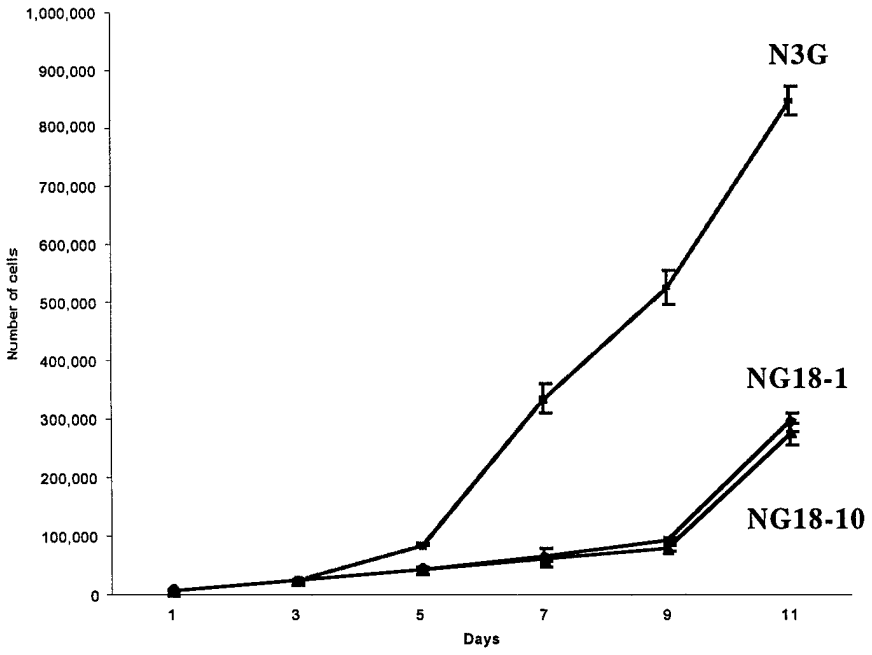

Figure 1. *Expression of the recombinant Hssh3bp1 slows growth of stably tranfected LnCaP cells.* Identical number of cells (10,000) were plated in triplicates in six-well plates. The growth of cells was monitored by counting the number of cells following the trypsin digestion as described (Schwab *et al.*, 2000). Note that the clones expressing the recombinant Hssh3bp1 cDNA, NG18-1, and NG18-10, grow much slower than the mock-transfected control, N3G-1. Averages and standard deviations were calculated for each time point.

Experimental problems and alternative strategies.

We attempted to perform soft agar assay using the above cloned LnCaP-Hssh3bp1 cell lines. No colony formation was observed even with the mock control. This suggested that LnCaP cells may not be suitable for this type of experiments. After repeating the attempt unsuccessfully for the second time we gave up these experiments as non-productive. To address these experiments we developed Hssh3bp1 stable clones of PC3 cells (another malignant tumor cell line) (Fig 2). This cell line is known to form colonies in soft agar and is tumorigenic in mice. Thus it is hypothesized that expression of Hssh3bp1 will slow growth of these cells and lower tumorigenicity in nude mice. We also plan to test whether overexpression Hssh3bp1 mutation found in LnCAP cell line (*Macoska et al.*, 2001) induces PC3 cells to grow faster. These experiments are consistent with Aim 2b.

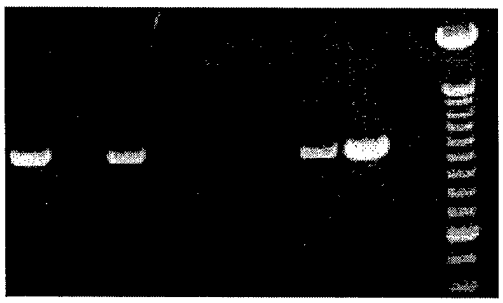

Figure 2. *RT-PCR screening of PC-3 clones stably expressing isoform 2 of Hssh3bp1.* Isoform 2 oligonucleotide primers specific for the recombinant Hssh3bp1cDNA were designed (a primer specific to the 3' end noncoding region of the Hssh3bp1 transcript present in the plasmid sequences determines specificity of the primer pair, data not shown) and used for screening of total RNA obtained form cultured PC3 cells. The cells were transfected with Hssh3bp1 in pEGFP plasmid (Clontech) after removal of GFP sequences. Clones were grown in tissue culture media containing neomycin for the period of two weeks by which all of control cells (i.e. wild type PC3) were killed off. PCR products obtained from PC3 clones were separated on 1% agarose gel containing ethidium bromide. *Lane 1*, cells transiently transfected with Hssh3bp1; *lane 2*, cells transfected with vector only (mock control); *lanes 3-7*, stable clones after selection; *lane 8*, control PCR (positive control) from LnCaP cells expressing Hssh3bp1 cDNA; *lane 9*, control PCR (negative control) from mock LnCaP cells. *Lane 10*, 100 bp ladder (Gibco-BRL). Please note that only two clones, lanes 3 and 7, are positive for expression of recombinant Hssh3bp1.

Progress towards Aim 3

The major goal of this aim is to identify potential signal transduction mechanism(s) involving Hssh3bp1. In Aim 3b we hypothesized that phosphorylation of Hssh3bp1 occurs following various treatments of cells. It was not known whether and by what enzyme Hssh3bp1 is phosphorylated. However, we hypothesized that Abl kinase is a candidate enzyme since it binds to Hssh3bp1 (Ziemnicka-Kotula *et al.*, 1998). We established that Hssh3bp1 is phosphorylated by Abl kinase *in vitro*. The major task now is to test whether Hssh3bp1 is phosphorylated in prostate cell lines and whether the phosphorylation is affected in response to growth factors. These studies may help us understand potential mechanism by which Hssh3bp1 regulates growth.

Hsshb3p1 is phosphorylated by Abl kinase in vitro (Fig 3). *In vitro* kinase assay. We established *in vitro* kinase assay to be used to determine Hssh3bp1 tyrosine phosphorylation by Abl kinase. Purified recombinant Abl kinase was purchased from New England Biolabs. *In vitro* translated Hssh3bp1 (Macoska *et al.*, 2001) was immunopreciptated with antibody Ab-2 as described (Xu *et al.*, 2000) in the presence of phosphatase inhibitors such as orthovanadate and β-glycerophosphate (Fig. 3a). Following 3x wash of the resin in the 1 X Abl kinase buffer (50 mM Tris HCL, 10 mM $MgCl_2$, 1 mM EGTA, 2 mM dithiotreitol, 0.01% Brij, PH 7.5) reactions were carried out in the buffer supplemented with the 100 μM ATP and ^{32}P γ-ATP to the final specific activity of 5000 μCi/μmol. Incubation was performed at 30°C for indicated times (Fig. 3b). Reactions were stopped by addition of SDS sample buffer and separated on SDS-Tricine gels, blotted onto the PVDF membranes. Phosphorylated bands were detected by autoradiography. Following

autoradiography the blot was incubated with mAb 4E2 to Hsshb3p1. As a control, a reaction with no Abl kinase present in the incubation buffer was used. In further experiments the quantity of incorporated ^{32}P into substrate bands will be determined by PhosphorImager analysis or by scintillation counter for quantitative phosphorylation assays; the positive control Abl peptide substrate will be used (EAIYAAPFAKKK, New Englnd Biolabs).

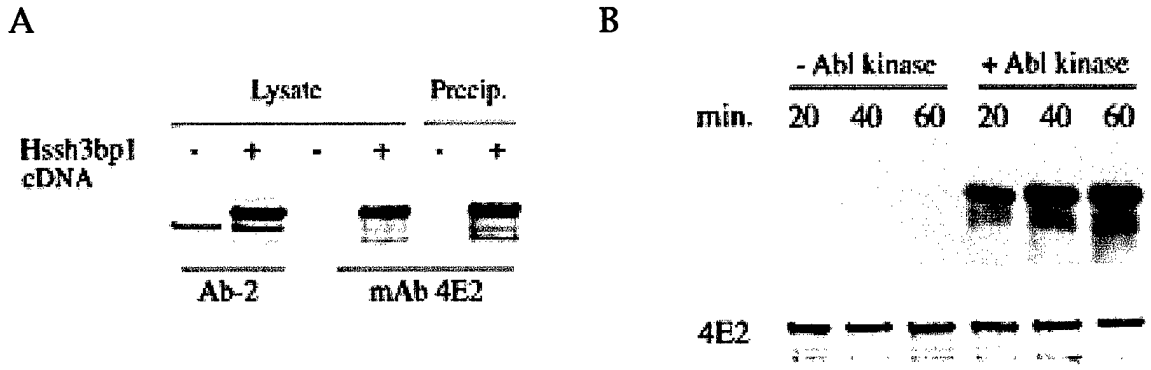

Figure 3. *Phosphorylation of Hsshb3p1 in vitro by Abl tyrosine kinase.* ***A,*** Following *in vitro* translation lysates (***Lysate***) containing,(+), and, lacking (-), Hsshb3p1 cDNA were blotted with antibodies to Hssh3bp1, Ab-2 (polyclonal, ***Ab-2***), and 4E2 (monoclonal, ***mAb 4E2***). Hssh3bp1 polypeptide, isoform 2, was precipitated from lysates with the polyclonal antibody Ab-2. Identical portions of resin containing the precipitated polypeptide (***Precip, +)*** were used as substrate in kinase assay (described above). ***B,*** *Top*, autoradiogram (performed at-70°C for 3hrs) of the western blot, *Bottom*, developed with mAb 4E2 to Hssh3bp1. Reactions were incubated with (+) or without (-) Abl kinase for indicated amount of time. Note increased intensity of the major band representing Hssh3bp1 with increased incubation time. Polypeptides were separated on SDS-Tricine polyacrylamide gels (7%) followed by blotting onto the PVDF membrane.

Key Research Accomplishements

- **Expression of Hssh3bp1 inhibits growth of the prostate tumor cell line LNCaP *in vitro.***
- **Establishement of Hssh3bp1 as a substrate of Abl tyrosine kinase *in vitro.***

Reportable Outcomes

Development of stable prostate cell lines expressing Hssh3bp1.

The LnCap and PC3 cell lines expressing Hssh3bp1 established in our laboratory will be available to scientific community upon publication of the results of this work.

Development of the NIH grant application entitled "Regulation of Macropinocytosis by Hssh3bp1"(R01 NS 044968-01) based on some of the results of this work.

The above grant received relatively high score and 25.7 percentile upon first submission. If required for funding consideration the grant will be resubmitted.

Conclusions

Two major conclusion of the presented progress of work are:

1. Expression of Hssh3bp1 inhibits *in vitro* growth of prostate tumor cell line LnCAP.

2. Hssh3bp1 is a substrate of Abl tyrosine kinase *in vitro*.

Observed inhibition of growth of LnCAP cells by expression Hssh3bp1 is critical in development of understanding the role of Hssh3bp1 in prostatic adenocarcinoma. However, we do not know the mechanism of the growth regulation by Hssh3bp1. Our *in vitro* data indicating that Hssh3bp1 may be a substrate for Abl kinase phosphorylation points to the possible role of Abl tyrosine kinase in this regulation. Established cell lines stably expressing Hssh3bp1may help to develop a detail understanding of this potential mechanism. Although most of these mechanistic studies are performed *in vitro* (in cultured cell lines) they are likely to provide a starting point for examination of Hssh3bp1 role in prostate tissue from patients.

Another important aspect of our studies in the nearest future will address potential tumor-inhibiting function of Hssh3bp1 in nude mice. This is a critical step of the proposed research and the PC3-Hssh3bp1 stable clones will be utilized in these experiments.

References

1. Macoska, J. A., Xu, J., Ziemnicka, D., Schwab, T., Rubin, M., and L. Kotula. 2001. Loss of expression of human spectrin Src homology domain binding protein 1 is associated with 10p loss in human prostatic adenocarcinoma. *Neoplasia* 3: 99-104.

2. Schwab , T.S., Steward, T., Lehr, J., Pienta, K.J., Rhim, J.S., and J. A. Macoska. 2000. Phenotypic characterizationof immortilized Normal and primary tumor-derived human prostate epithelial cell cultures. *The Prostate* 44: 164-171.

3. Xu, J., Ziemnicka, D., Merz, G.S., and L. Kotula. 2000. Human spectrin SH3 domain binding protein 1 regulates macropinocytosis in NIH 3T3 cells. *J Cell Sci.* 113:3805-3814.

4. Xu, J., Ziemnicka, D., Scalia, J., and L. Kotula. 2001. Monoclonal antibodies to α spectrin Src homology domain 3 associate with macropinocytic vesicles in nonerythroid cells. *Brain Res.* 898:171-177.

5. Ziemnicka-Kotula, D., Xu, J., Gu, H., Potempska, A., Kim, K. S., Jenkins, E. C., Trenkner E., and L. Kotula. 1998. Identification of A Candidate Spectrin SH3 Binding Protein Suggests A General Mechanism of Association of Tyrosine Kinases with the Spectrin-Based Membrane Skeleton. *J. Biol. Chem.* 273: 13681-13692.

Appendices - None